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THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS *US60/545,382*

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

DETECTION AND QUANTIFICATION OF RARE MUTATIONS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 20 ☐ CD(s), Number _____

☒ Drawing(s) *Number of Sheets* 1 ☐ Other (specify) _____

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees.

☒ The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-0850 - Nixon Peabody (charge fee deficiencies)

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FILING FEE Amount ($): 80.00

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Respectfully submitted,

SIGNATURE Ronald I Eisenstein

Date 2/18/04

TYPED or PRINTED NAME Ronald I. Eisenstein

REGISTRATION NO. 30,628 *(If appropriate)*

Docket Number: 701586-54980-P

TELEPHONE 617-345-6054

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PTO/SB/17 (10-03)
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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

[X] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	To be assigned
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First Named Inventor	Charles R. Cantor
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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =	X	=
Independent Claims		- 3** =	X	=
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

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3. ADDITIONAL FEES

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1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBMITTED BY				(Complete *(if applicable)*)	
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Docket No. 701586-54980-P PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Charles R. Cantor and Chunming Ding
Serial No.: To be assigned Group: To be assigned
Filed: To be assigned Examiner: To be assigned
For: DETECTION AND QUANTIFICATION OF RARE MUTATIONS

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
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are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10, on the date indicated above and is addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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METHOD FOR DETECTING AND QUANTIFYING RARE MUTATIONS/POLYMORPHISMS

BACKGROUND OF THE INVENTION

[001] The present invention relates to methods of detecting and quantifying rare nucleic acids changes, mutations or polymorphisms in a nucleic acid sample, that is, the sample contains a much smaller percentage of the changed, mutated or polymorphic nucleic acid molecule compared to that of the wildtype or more common variant or a control nucleic acid molecule.

[002] Detection of a nucleic acid containing a rare polymorphism or mutation can be problematic. Such problems occur in numerous situations, for example, if a nucleic acid sample is suspected of containing a small population of mutant nucleic acids such as in diagnosis or prognosis of cancer, viral infections, variations in viral infections, such as various HIV strains in the same individual, and the like. In all these cases, it is important to know accurately whether the nucleic acid sample actually contains the rare mutant allele or not, and in many cases it would be helpful to know how much mutant allele containing nucleic acid is present in the sample, particularly in relation to the wildtype or the more common nucleic acid molecule.

[003] Methods for detection and quantification of nucleic acids that contain differences which are present in only at low quantities or small percentages compared to a wildtype or control nucleic acid molecule in the sample can be important in many clinical applications. Non-limiting examples of applications wherein detection of rare nucleic acid changes would be useful include early benign or malignant tumor detection, prenatal diagnostics particularly when using a plasma or serum DNA sample from the mother, early viral or bacterial disease detection, environmental monitoring, monitoring of effects of pharmaceutical interventions such as early detection of multi drug resistance mutations in cancer treatment. Also, a number of mutations causing inherited diseases result in reduction of the transcript levels. Therefore, improved methods allowing detection of the mutant transcript which is present at very low levels would allow a

simplification of mutation detection, particularly at the RNA level, in cases wherein the mutant transcript levels are low.

[004] Detection of rare mutations could also provide tools for forensic nucleic acid sample analysis by providing a system to reliably detect presence or absence of specific nucleic acid polymorphisms to provide evidence to exonerate a crime suspect.

[005] Additionally, detection of rare mutations in biological agents such as bacteria and viruses that can be used as a biological warfare agents would provide an important tool for detecting spread of harmful biological materials.

[006] Another problem requiring a satisfactory solution is in the commonly used genotyping methods, is a so called "allele dropout" -problem which happens when one allele is poorly amplified or detected and a heterozygotic allele is mis-called as a homozygote. The dropout allele is usually, but not always, the allele that produces a higher molecular weight base extension product. A method which would allow addressing the allele dropout, particularly in clinical diagnostic applications, would be extremely useful and improve the accuracy of distinguishing heterozygotes from homozygotes, which can be crucial for evaluating, for example, disease prognosis.

[007] The methods for mutation detection and nucleic acid molecule quantification have traditionally included Southern-blot and Northern-blot hybridization, ribonuclease protection assay, and polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR) based methods. However, both direct detection methods and PCR-based methods to detect nucleic acid molecules suffer from lack of sensitivity to detect or amplify the rare nucleic acid mutation, when the sample nucleic acid contains both a large amount of the wildtype nucleic acids and a much smaller amount of the rare mutation or polymorphism.

[008] Absolute quantification of nucleic acid molecule copy numbers in a sample is a requirement if one wishes to monitor the number of mutant or polymorphic nucleic acids, for example, at different time points or as a response to a pharmaceutical intervention. However, quantification of nucleic acid copy numbers for rare mutations is difficult using PCR based methods because the common nucleic acid molecule is also amplified exponentially and the mixture of amplified sample almost always contains

large amounts of the wildtype or "normal" nucleic acid variant relative to the rare nucleic acid variant.

[009] A number of quantitative PCR based methods have been described including RNA quantification using PCR and complementary DNA (cDNA) arrays (Shalon et al., Genome Research 6(7):639-45, 1996; Bernard et al., Nucleic Acids Research 24(8):1435-42, 1996), solid-phase mini-sequencing technique, which is based upon a primer extension reaction (U.S. Patent No. 6,013,431, Suomalainen et al. Mol. Biotechnol. Jun;15(2):123-31, 2000), ion-pair high-performance liquid chromatography (Doris et al. J. Chromatogr. A May 8;806(1):47-60, 1998), 5' nuclease assay or real-time RT-PCR (Holland et al. Proc Natl Acad Sci USA 88: 7276–7280, 1991), and real competitive RT-PCR (Ding et al. Proc Natl Acad Sci USA 100:3059-3064, 2003.

[010] Methods using PCR and internal standards differing by length or restriction endonuclease site from the desired target sequence allowing comparison of the standard with the target using gel electrophoretic separation methods followed by densitometric quantification of the target have also been developed (see, e.g., U.S. Patent Nos. 5,876,978; 5,643,765; and 5,639,606. These methods, also sometimes referred to as StaRT-PCT, have severe limitations in measuring an absolute transcript quantity in a biological sample. Because of the size differences between the standard and the target sequence, the PCR amplification can not be expected to be the same for both the standard and the target sequence. Further, because a separate gel electrophoretic separation and/or restriction endonuclease digestion followed by gel electrophoretic separation, and densitometric measurement are required after amplification, the method has steps which are prone to errors and make the quantification of small amounts of nucleic acids cumbersome.

[011] Therefore, it would be useful to develop a method which allows sensitive and accurate detection and quantification of nucleic acids containing rare changes and which can be easily automated and scaled up to accommodate testing of large numbers of sample and which overcomes the sensitivity problems of nucleic acid detection. Such a method would enable diagnosing different pathological conditions, including viruses, bacteria and parasites, as well as different benign and malignant tumors, neurological disorders, heart disease and autoimmune disorders. Such a method would also allow

quantifying the rare transcripts of interest for diagnostic, prognostic and therapeutic purposes.

SUMMARY OF THE INVENTION

[012] The present invention is directed to a method for detecting and quantifying rare mutations in a nucleic acid sample. The nucleic acid molecules under investigation can be either DNA or RNA. The rare mutation can be any type of functional or non-functional nucleic acid change or mutation, such as deletion, insertion, translocation, inversion and one or more base substitution. Therefore, the methods of the present invention are useful in detection of rare mutations in, for example, diagnostic, prognostic and follow-up applications, when the targets are rare known nucleic acid variants mixed in with the wildtype or the more common nucleic acid variant(s).

[013] In one embodiment, the invention provides a method of detecting nucleic acids with a rare mutation comprising the steps of amplifying a nucleic acid molecule with at least two primers flanking the mutation site, designing a detection primer so that the 3' end of the detection primer is immediately adjacent to the first nucleic acid which differentiates the wildtype nucleic acid variant from the mutant nucleic acid variant molecule, removing the excess dNTPs after the amplification reaction, performing a primer extension reaction using the detection primer and at least one dNTP or ddNTP, which corresponds to a nucleoside adjacent to the detection primer in the rare mutant nucleic acid molecule, wherein the presence of a primer extension product in the reaction indicates the presence of the nucleic acid with a rare mutation. In the preferred embodiment, only one dNTP or ddNTP is used. However, so long as the primer is designed so that the background wildtype or the more common nucleic acid molecule(s) cannot serve as a template, more than one dNTP/ddNTP can be used in the primer extension reaction.

[014] In one embodiment, the invention provides a method, wherein only one dNTP, which corresponds to the nucleotide adjacent to the detection primer in the rare mutant nucleic acid molecule is used together with the detection primer.

[015] In another embodiment, the invention provides a method of detection nucleic acid molecules with a rare mutation comprising amplifying the nucleic acid sample with two primers that are designed to allele-specifically amplify the rare mutation containing

nucleic acid, removing the excess dNTPs from the reaction after the amplification reaction, performing the primer extension reaction with at least one dNTP or ddNTP, preferably dNPT, and a detection primer, which has been designed so that the 3' end is immediately adjacent to the mutation site, so that only the mutant nucleic acid will serve as a template to the primer extension reaction when the corresponding dNTP or ddNTP is used, and detecting the primer extension reaction product, wherein presence of the primer extension product after the primer extension reaction indicates the presence of a nucleic acid with a rare mutation.

[016] In one embodiment, two reactions are performed using two different detection primers, wherein the first detection primer is designed to amplify the sense strand so that the 3' end of the primer anneals immediately adjacent to the mutation site in the sense strand and in the second reaction the detection primer is designed to amplify the antisense strand so that the 3' end of the primer anneals immediately adjacent to the mutation site in the antisense strand.

[017] In yet another embodiment, the invention provides a method of quantifying nucleic acid molecules with rare mutations comprising the steps of amplifying a nucleic acid sample and a known amount of a control nucleic acid sample in the same reaction, wherein the control nucleic acid sample has been designed to have the same sequence as the rare mutation containing amplicon with the exception of at least one, 2, 3, 4, 5-10, preferably only one nucleic acid difference immediately adjacent to the mutation site. The amplification is performed with primers flanking the mutation site. After amplification, the excess dNTPs are removed and a primer extension reaction is performed using at least one detection primer, which is designed so that the 3' end of the primer anneals immediately adjacent to the rare mutation site. The detection reaction is performed in the presence of dNTPs and/or ddNTPs. For example, at least one deoxynucleotide (dNTP), corresponding to the mutant nucleoside immediately 3' of the detection primer and two dideoxynucleotides (ddNTPs), which correspond to the nucleoside(s) that differentiate the control from the rare mutant nucleic acid. The primer extension products are then detected, and because the amount of the control originally added to the amplification reaction is known, the ratio of the control and the rare mutant containing nucleic acid molecules is used to determine the exact quantity of the mutant

nucleic acid molecules in the sample. Preferably, only one dNTP is used. However, so long as the primer and dNTP/ddNTP combinations are designed so that the more common nucleic acid cannot be amplified in the primer extension reaction, the combination of dNTPs/ddNTPs may vary.

BRIEF DESCRIPTION OF THE FIGURE

[018] Figures 1A-1E show quantification of rare mutations. In the absence (Figure 1A), 20 fold excess (Figure 1C) and 100 fold excess (Figure 1D) of wild type DNA, the ratios of mutant DNA and the competitor DNA are very similar. In Figure 1B, only 500 fold excess wild type DNA was present and neither mutant nor competitor DNA was present. In Figure 1E, 500 fold excess wild type DNA, mutant DNA and competitor DNA were all present. The sequences of the nucleic acid molecules shown in the Figure 1 are described in the Example.

DETAILED DESCRIPTION OF THE INVENTION

[019] The present invention is directed to a method for detecting and quantifying rare mutations in a biological sample. The sample nucleic acid molecules that can be used in the methods of the present invention include DNA, RNA and cDNA molecules. The present invention provides a method for robustly detecting whether such rare mutations occur in a biological sample.

[020] The term "mutation" as used throughout the specification is intended to encompass any and all types of functional and/or non-functional nucleic acid changes, mutations or polymorphisms in the target nucleic acid molecule when compared to a wildtype variant of the same nucleic acid region or allele or the more common nucleic acid molecule present on the sample. Such changes, include, but are not limited to deletions, insertions, translocations, inversions, and base substitutions of one or more nucleotides.

[021] The term "rare mutation" as used herein and throughout the specification is intended to describe a mutation in a nucleic acid molecule which is present in less than 40% of the nucleic acid molecules in the sample, preferably in less than 30%, 20%, 15%, 10%, 8%, 5%, 4%, 3%, 2%, 1%, 0.8%, 0.5%, 0.1%, 0.05%, 0.01, or less compared to one

or more, more common nucleic acid variants, which are referred to throughout the specification as the "wildtype" nucleic acid variants. In one embodiment, the rare nucleic acid is present in the sample in amount less than 10%, preferably less than 1%. The sample may include one or more rare mutations and there may also be one or more wildtype variants in the nucleic acid sample.

[022] The deoxynucleotides or dNTPs according to the present invention are dATP, dTTP, dCTP, or dTTP. The dideoxynucleotides or the terminator nucleotides (ddNTPs) are ddATP, ddTTP, ddCTP, or ddTTP. The dNTPs and ddNTPs can also be labeled with, for example, different fluorescent dyes, or other labels, such as radioactive molecules, which do not interfere with the DNA polymerase function in the primer extension reaction. Differentially labeled dNTPs or ddNTPs can be used to differentiate the alleles after the primer extension reaction. Such labels and the methods of preparing labeled dNTPs and ddNTPs are well know to one skilled in the art.

[023] The terms "nucleic acid sample", "nucleic acid molecule", or "nucleic acid" as described throughout the specification are intended to encompass nucleic acids isolated from any biological material, e.g., human, animal, plant, bacteria, fungi, protist, viruses, from tissues including blood, hair follicles, or other tissues, such as skin biopsies, cells or cell cultures, body excrements such as semen, saliva, stool, urine, amniotic fluid and so forth. The nucleic acids can also be isolated from foodstuff, drinks, clothes, soil and any other source, wherein detection of rare nucleic acids compared to a more common or wildtype variants of the same is needed. Nucleic acid molecules can be isolated from a particular biological sample using any of a number of procedures, which are well-known in the art, the particular isolation procedure chosen being appropriate for the particular biological sample.

[024] In one embodiment, the invention provides a method of detecting one or more nucleic acids with a rare mutation comprising the steps of amplifying a nucleic acid molecule with two primers flanking or surrounding the mutation site, designing a detection primer so that the 3' end of the detection primer anneals immediately adjacent to a nucleic acid which is different in the mutant molecule compared to the more common wildtype variant of the same nucleic acid molecule, removing the excess dNTPs after the amplification reaction, performing a primer extension reaction using the

detection primer and at least one dNTP or ddNTP, which corresponds to the nucleotide adjacent to the detection primer present in the rare mutant nucleic acid molecule and is not present in the background of the more common nucleic acid molecule(s) or variant(s), wherein the presence of a primer extension product in the reaction indicates the presence of the nucleic acid with a rare mutation. Preferably, only one dNTP or ddNTP is used in the primer extension reaction.

[025] In one embodiment, the invention provides a method, wherein only one dNTP, which corresponds to the nucleoside adjacent to the detection primer in the rare mutant nucleic acid molecule is used together with the detection primer.

[026] For example, a nucleic acid molecule contains the following sequence:

[027] 5'TGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGG CAGGTTaggggcagatagcagtga[A/T]GAGAGCGAGAGAGCCATCTATTGCTTACATT TGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGC ACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTG3' [SEQ ID NO: 1],

[028] wherein [A/T] represents a base A to T mutation. T mutation occurs at low frequencies, for example, less than about 30%, 20%, 15%, 10%, 8%, 5%, 4%, 3%, 2%, 1%, 0.8%, 0.5%, 0.1%, 0.05%, 0.01% or less. Therefore, in a biological sample, most nucleic acid molecules for the region depicted above have the A base and only a very small percentage of the nucleic acid molecules present in the biological sample have the T base at the polymorphic site.

[029] To detect the rare mutation, nucleic acids are isolated from the source material, such as tissues/cells/fluids or other sources of interest, using any of the widely adopted methods well known to one skilled in the art. Two PCR primers flanking the mutation site are shown as underlined sequences in the above example, wherein only the sense strand is shown. The primers are designed to amplify the DNA region for both the wild type and mutant DNAs. To further increase the sensitivity of the method to detect rare known mutations, allele specific primers can be used to preferentially amplify the rare mutation containing nucleic acid molecule(s). After the PCR, the excess nucleotides in the amplification reaction are removed, for example, using a shrimp alkaline phosphatase or a spin column or any other method well known to one skilled in the art. A third primer, so called detection primer, which is shown in the above example in small

letters, is used in a base extension reaction. The third primer can also be designed from the opposite direction and the two primers in two parallel reactions can be used to cross-validate the results. It is important that the detection primer is designed so that its 3' end anneals immediately before a nucleoside which is different in the rare mutant(s) compared to more common wildtype nucleic acid variant(s) because the methods of the present invention are based on the premise of preferentially detecting the rare nucleic acid molecules.

[030] In the base extension reaction using the above presented example nucleic acid template, only ddTTP or alternatively, only dTTP is used so that only the mutant nucleic acid will be used as the template for the base extension reaction. The detection of the oligonucleotide resulting from the primer extension reaction, i.e., aggggcagatagcagtga-ddT [SEQ ID NO.: 2] indicates that the mutant allele is present.

[031] Other combinations of ddNTP and dNTP can also be used as far as the wild type nucleic acid(s) cannot be used as the template for the base extension reaction.

[032] The methods of the present invention can additionally be used to detect more than one rare nucleic acid variant in the sample. For example, a multiplex PCR and a subsequent multiplex primer extension reaction can be designed using the teachings of the present invention to detect at least 2, 3, 4, 5, 6, 7, 8, 9, 10-15 or even more than 15 mutations in the same reaction, as far are none of the wildtype, or more common variants of the respective nucleic acid targets can serve as a template for the detection primers in the primer extension reaction.

[033] The methods of the present invention are useful, for example, in detecting a small population of nucleic acids with a known mutation among a background of wildtype nucleic acid variants in, for example, early diagnosis or prognosis of cancer or malignant cell growth in an individual. The methods of the present invention are also useful in providing a means for early detection of malignant cells containing new or additional mutations which may be a result of treatment of the malignancies, such as appearance of multi drug resistance mutations in an individual with the proviso that these mutations are known or become known through screening of new mutations before designing the detection primers.

[034] The methods of the present invention also provide a useful tool to detect viral infections or emerging virus mutants in an individual infected with a virus, such as human immune deficiency virus (HIV), during the treatment of the disease thereby allowing early adjustment in treatment as a response to occurrence of new virus mutations.

[035] Due to their sensitivity, the methods of the present invention provide an ideal tool to detect rare mutations in detection of the presence and quantification of the amount of the rare nucleic acid changes. Therefore, applications for the methods of the present invention include, for example, early benign or malignant tumor detection, prenatal diagnostics particularly when using a blood sample from the mother, early viral or bacterial disease detection or detection of emerging strains of treatment resistant strains of bacteria or viruses in a target sample, environmental monitoring, monitoring of effects of pharmaceutical interventions such as early detection of multi drug resistance mutations in cancer treatment. The methods of the present invention are also useful in detection of rare mutant nucleic acid populations in mosaic organisms or individuals or one of their tissues composed of cells of more than one genotype, for example, in diagnosis of mitochondrial diseases or inherited diseases, wherein the mutation occurred after fertilization during early development of the embryo or fetus resulting in a mosaic genotype and consequently a mosaic phenotype.

[036] The methods of the present invention are also useful in detection of rare mutations in inherited diseases which result in reduction of the transcript levels. It is sometimes easier to detect mutations from an RNA sample than from a genomic DNA sample. However, mutations causing significantly reduced transcript levels are often missed in these screens. The methods of the present invention can be used in detecting the known transcript reducing mutations which can be considered "rare mutations" because the mutant transcript population represents only a small percentage of the nucleic acids in the target sample.

[037] Detection of rare mutations using the methods of the present invention also provide tools for forensic nucleic acid sample analysis by providing a system to reliably detect presence or absence of specific known nucleic acid polymorphisms to provide evidence, for example, to include or exclude crime suspects.

[038] Additionally, the methods of the present invention are useful in detection of rare extremely virulent or dangerous mutations in biological agents, such as bacteria and viruses, that can be used as a biological warfare agents. As the knowledge of the dangerous mutations in viruses and/or bacteria increases, the present invention provides methods to detect small quantities of these abnormal mutants in a larger population of wildtype or less virulent agents.

[039] The present invention also provides that the method can be modified for genotyping assays that might have an allele dropout problem. An allele dropout occurs when one allele is poorly amplified or detected, and a heterozygotic allele is mis-called as a homozygote. The dropout allele is often, but not always, the allele that produces a higher molecular weight base extension product.

[040] For example, if the allele with the T base at the SNP (single nucleotide polymorphism) site is dropped out in a typical genotyping assay in the above presented example nucleic acid, the method according to the present invention provides that the use of ddTTP only, or ddTTP and much lower concentrations of other ddNTP/dNTP combinations, for the base extension reaction, will result in preferential extension of the 'dropped-out' allele, and therefore allele dropout is avoided.

[041] The detection methods for detecting the primer extension products of the present invention can be any detection method which is capable of detecting the primer extension product after the primer extension reaction. If the dNTP or ddNTP is labeled with a detectable marker such as a fluorescent or radioactively label or some other detectable chemical group, the detection method is based on detecting the incorporation of the label into the primer extension product. Such detection methods include gel electrophoresis with laser detection or gel electrophoresis with detection of radioactivity, or other methods well known to one skilled in the art. The preferred method for detecting the primer extension products comprising the rare mutant nucleic acid is MALDI-TOF MS, using e.g. MASSARRAY^{TM} system (Sequenom Inc., San Diego, CA).

[042] In another embodiment, the invention provides a method of detection nucleic acid molecules with a rare mutation comprising amplifying the nucleic acid sample with two primers that are designed to allele-specifically amplify the rare mutation containing nucleic acid, removing the excess dNTPs from the reaction after the amplification

reaction, performing the primer extension reaction with only one dNTP or ddNTP, preferably dNPT, and a detection primer, which has been designed so that the 3' end is immediately adjacent to the mutation site, so that only the mutant nucleic acid will serve as a template to the primer extension reaction when the corresponding dNTP or ddNTP is used, and detecting the primer extension reaction product, wherein presence of the primer extension product after the primer extension reaction indicates the presence of a nucleic acid with a rare mutation.

[043] In one embodiment, two reactions are performed using two different detection primers, wherein the first detection primer is designed to amplify the sense strand so that the 3' end of the primer anneals immediately adjacent to the mutation site in the sense strand and in the second reaction the detection primer is designed to amplify the antisense strand so that the 3' end of the primer anneals immediately adjacent to the mutation site in the antisense strand.

[044] In yet another embodiment, the invention provides a method of quantification of nucleic acid molecules with rare mutations comprising the steps of amplifying a nucleic acid sample and a known amount of a control nucleic acid sample in the same reaction, wherein the control nucleic acid sample has been designed to have the same sequence as the rare mutation containing amplicon with the exception of one nucleic acid difference immediately adjacent to the mutation site. The amplification is performed with primers flanking the mutation site. After the amplification, the excess of dNTPs are removed and a primer extension reaction is performed using a detection primer, which is designed so that the 3' end of the primer anneals immediately adjacent to the rare mutation site. The detection reaction is performed in the presence of one deoxynucleotide (dNTP) and two dideoxynucleotides (ddNTPs): the dNTP corresponds to the first nucleoside after the 3' end of the detection primer in the nucleic acid with the rare mutation, the first ddNTP corresponds to the nucleoside artificially created to the control which differs from the nucleoside present in the rare mutant allele, and the second ddNTP corresponds to the nucleoside present in the rare mutant allele, preferably immediately after the mutation site. The primer extension products are then detected, and because the amount of the control originally added to the amplification reaction is known,

the ratio of the control and the rare mutant containing nucleic acid molecules is used to determine the exact quantity of the mutant nucleic acid molecules in the sample.

[045] The standard nucleic acid can be prepared using any method of nucleic acid synthesis know to one skilled in the art, including, for example, chemical oligonucleotide synthesis, by cloning and targeted mutagenesis, or by PCR with mutagenized primers.

[046] The methods of the present invention can be modified to utilize one or more control or competitor nucleic acids to quantify the amount of one or more rare mutant nucleic acid molecules in the same reaction.

[047] The amount of the primer extension products is consequently measured by any of a variety of means, preferably by Mass Spectrometry (MALDI-TOF, or Matrix Assisted Laser Desorption Ionization – Time of Flight). In MALDI-TOF mass spectrometry, the peak area ratio between the products from the standard and the nucleic acid of interest comprising the rare mutation represents the ratio of the standard and the gene of interest. Since the concentration of the standard is known, the concentration of the nucleic acids with the rare mutation can be calculated.

[048] Products of the primer extension reaction are detected and quantified using methods including, but not limited to, MALDI-TOF mass spectrometry, PYROSEQUENCING™, real time PCR, hybridization-based techniques, third wave invader assay, and fluorescence-based detection techniques.

[049] In one preferred embodiment, the detection of the primer extension products in the methods of the present invention is performed using the MALDI-TOF mass spectrometry, using, for example the MASSARRAY™ system according to the manufacturer's instructions (Sequenom Inc., San Diego, CA).

[050] Alternatively, an INVADER® assay can be used (Third Wave Technologies, Inc (Madison, WI)). This assay is generally based upon a structure-specific nuclease activity of a variety of enzymes, which are used to cleave a target-dependent cleavage structure, thereby indicating the presence of specific nucleic acid sequences or specific variations thereof in a sample (see, e.g. U.S. Patent No. 6,458,535). For example, an INVADER® operating system (OS), provides a method for detecting and quantifying DNA and RNA. The INVADER® OS is based on a "perfect match" enzyme-substrate reaction. The INVADER® OS uses proprietary CLEAVASE® enzymes (Third Wave

Technologies, Inc (Madison, WI)), which recognize and cut only the specific structure formed during the INVADER® process. The INVADER® OS relies on linear amplification of the signal generated by the INVADER® process, rather than on exponential amplification of the target. This allows quantification of target concentration.

[051] In the INVADER® process, two short DNA probes hybridize to the target to form a structure recognized by the CLEAVASE® enzyme. The enzyme then cuts one of the probes to release a short DNA "flap." Each released flap binds to a fluorescently-labeled probe and forms another cleavage structure. When the CLEAVASE® enzyme cuts the labeled probe, the probe emits a detectable fluorescence signal.

[052] In one embodiment, the primer extension products for the rare mutations are detected using PYROSEQUENCING™ (Uppsala, Sweden), which is essentially sequencing by synthesis. A sequencing primer, designed to anneal directly next to the nucleic acid differing between the rare and the common allele or the artificially produced quantification standard is first hybridized to a single stranded, PCR amplified, DNA template comprising both the target and the standard PCT product, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5′ phosphosulfate (APS) and luciferin. One of four deoxynucleotide triphosphates (dNTP), for example, corresponding to the nucleotide present in the standard template, is then added to the reaction. DNA polymerase catalyzes the incorporation of the dNTP into the standard DNA strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. Consequently, ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5′ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a PYROGRAM™. Each light signal is proportional to the number of nucleotides incorporated and allows determination of the amount of the standard nucleic acid sequence. Thereafter, apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete, another dNTP is added which corresponds to the dNTP present in the target template the amount of which

is to be determined. Finally, addition of dNTPs is performed one at a time. Deoxyadenosine alfa-thio triphosphate (dATPαS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. Because the amount of the standard added in the PCR is known, the amount of the target can be calculated from the ratio of the incorporated dNTPs. For detailed information about reaction conditions, see, e.g. U.S. Patent No. 6,210,891, which is herein incorporated by reference in its entirety.

[053] The following illustrates quantification of concentration or copy numbers of rare alleles using the methods of the present invention. The sequence is the same example as above:

[054] 5'TGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTaggggcagatagcagtga[A/T]{G/C}AGAGCGAGAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTG3' [SEQ ID NO: 12],

[055] wherein all the notations are the same as above, except the {G/C}. The G/C mutation is created to provide a detectable standard for the quantification reaction. In other words, a synthetic oligonucleotide with the sequence as the following

[056] GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTAGGGGCAGATAGCAGTGATCAGAGCGAGAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACC [SEQ ID NO.: 3] is used as the internal standard for competitive PCR, wherein the bolded, underlined T represents the same nucleoside as in the rare mutant nucleic acid and the C is created to provide a detectable difference between the rare mutant and the standard.

[057] The competitor carries the T base as the rare mutation at the natural polymorphic site. In addition, it also has a C base, instead of the G base, at the position next to the polymorphic site.

[058] PCR, excess dNTP removal using, for example, shrimp alkaline phosphatase treatment, and consequently the base extension reaction are carried out. In the base extension reaction of the example, dTTP, ddGTP and ddCTP mixture is used. As a result, two extension products: aggggcagatagcagtgaTddG [SEQ ID NO.: 4] and

aggggcagatagcagtgaTddC [SEQ ID NO.: 5] are produced. The first product comes from the rare mutation and the second product comes from the internal standard, the initial concentration of which is known. The ratio of the two products can be quantified by, for example, MALDI-TOF mass spectrometry, or other techniques, such as fluorescence measurement when ddCTP and ddGTP are tagged with different fluorescent groups.

EXAMPLE

[059] **Detection and quantification of rare mutation.** Three DNA sequences including wild type (wt), mutant (mut) and a competitor or the standard were used in this experiment. The sequences were:

[060] WILD TYPE:

[061] 5'GTGGCAGATCTCTTCATGGTCTTCGGTGGCTTCACCACCAACCTC TACACCTCTCTCCATGGGTACTTCGTCTTTGG-3' [SEQ ID NO.: 6]

[062] MUTANT:

[063] 5'GTGGCAGATCTCTTCATGGTCTTCGGTGGCTTCACCACCATCCTC TACACCTCTCTCCATGGGTACTTCGTCTTTGG-3' [SEQ ID NO.: 7]

[064] COMPETITOR:

[065] 5'GTGGCAGATCTCTTCATGGTCTTCGGTGGCTTCACCACCATGCTC TACACCTCTCTCCATGGGTACTTCGTCTTTGG-3' [SEQ ID NO.: 8]

[066] The competitor was used as an internal standard for mut DNA quantification. Wt DNA is used as the background DNA which exist at a much higher concentration than mut DNA. The PCR primer sequences are:
5'ACGTTGGATGTGGCAGATCTCTTCATGGTC-3' [SEQ ID NO.: 9] and
5'ACGTTGGATGCCAAAGACGAAGTACCCATG-3' [SEQ ID NO.: 10]. The extension primer sequence was 5'CGGTGGCTTCACCACCA-3' [SEQ ID NO.: 11]. The extension ddNTP/dNTP mixture was dTTP/ddGTP/ddCTP.

[067] Different mixtures of the three DNAs were co-amplified by PCR. Excess dNTPs used in the PCR reaction were removed by shrimp alkaline phosphatase. Primer extension reaction was carried out using the extension primer and the extension ddNTP/dNTP mixture. Figures 1A-1E illustrate the results of the MALDI-TOF mass spectrometric analysis of the primer extension products. In the absence of wild type

DNA (Figure 1A), 20 fold excess of wild type DNA (Figure 1C) and 100 fold excess of wild type DNA (Figure 1D), the ratios of mutant DNA and the competitor DNA are very similar which well exemplifies that the method of the present invention is capable of specifically amplifying the mutant allele and that the rare mutation can be enriched to provide an efficient detection and quantification method for detecting rare mutations in the presence of the much more common background nucleic acid variant. In Figure 1B, only 500 fold excess wild type DNA was present and neither mutant nor competitor DNA was present. The figure illustrates the specificity of the system to amplify only the rare mutant and the added standard, or competitor nucleic acid. In Figure 1E, 500 fold excess wild type DNA, mutant DNA and competitor DNA were all present.

[068] The references cited throughout the specification are herein incorporated by reference in their entirety.

CLAIMS

1. A method of detecting nucleic acids with a rare mutation comprising the steps of;

a) amplifying a nucleic acid molecule with primers flanking the rare mutation site(s);

b) removing the excess dNTPs after the amplification reaction;

c) performing a primer extension reaction using a detection primer(s) which is designed so that the 3' end of the detection primer is immediately adjacent to a nucleic acid which differentiates the wildtype from the mutant nucleic acid molecule, and at least one dNTP or ddNTP, which corresponds to a nucleoside adjacent to the detection primer in the rare mutant nucleic acid molecule; and

d) detecting the presence of the primer extension product(s) after the primer extension reaction and/or the consumption of dNTP,

wherein the presence of a primer extension product in the reaction or the consumption of dNTP indicates the presence of the nucleic acid with a rare mutation.

2. The method of claim 1, wherein the consumption of dNTP is detected using pyrosequencing.

3. The method of claim 1, wherein only one dNTP or ddNTP corresponding to a nucleoside differentiating the rare nucleic acid variant from the more common nucleotide variant(s) is used .

4. The method of claim 3, wherein only one dNTP is used.

5. The method of claim 1, wherein a mixture of dNTP(s)/ddNTP(s)ddNTP(s) are used, wherein none of the dNTPs or ddNTPs can also be used for the extension of the wildtype DNA.

6. The method of claims 1, 3, 4 and 5, wherein the production of a primer extension product in the reaction is looked for.

7. A method of detecting nucleic acid molecules with a rare mutation comprising the steps of:

a) amplifying the nucleic acid sample with primers that are designed to allele-specifically amplify the rare mutation containing nucleic acid;

b) removing the excess dNTPs from the reaction after the amplification reaction;

c) performing the primer extension reaction with at least one dNTP or ddNTP, preferably dNTP, and a detection primer(s), which has been designed so that the 3' end anneals immediately adjacent to the mutation site, so that only the mutant nucleic acid will serve as a template to the primer extension reaction when the corresponding dNTP(s) or ddNTP(s) are used; and

d) detecting the primer extension reaction product(s),

wherein presence of the primer extension product(s) after the primer extension reaction indicates the presence of a nucleic acid with a rare mutation.

8. The method of claims 1, 2, 3, 4, 5, 6 or 7, wherein parallel primer extension reactions are performed using two different detection primers, wherein the first detection primer is designed to amplify the sense strand so that the 3' end of the primer anneals immediately adjacent to the mutation site in the sense strand and in the second reaction the detection primer is designed to amplify the antisense strand so that the 3' end of the primer anneals immediately adjacent to the mutation site in the antisense strand.

9. A method of determining the concentration or the copy number of nucleic acid molecules with rare mutations comprising the steps of:

a) amplifying a nucleic acid sample and a known amount of a control competitive nucleic acid standard sample in the same reaction, wherein the control nucleic acid sample has been designed to have the same sequence

as the rare mutation containing amplicon with the exception of one nucleic acid difference immediately adjacent to the mutation site, with primers flanking the mutation site;

b) removing the excess dNTPs;

c) performing a primer extension reaction using a detection primer(s), which is designed so that the 3' end of the primer anneals immediately adjacent to the rare mutation site and in the presence of at least one deoxynucleotide (dNTP) and two dideoxynucleotides (ddNTPs), wherein the dNTP corresponds to the first nucleoside after the 3' end of the detection primer in the nucleic acid with the rare mutation, the first ddNTP corresponds to the nucleoside artificially created to the control which differs from the nucleoside present in the rare mutant allele, and the second ddNTP corresponds to the nucleoside present in the rare mutant allele immediately after the mutation site;

d) detecting the production of primer extension products and/or consumption of ddNTP; and

e) determining the ratio of the amplified rare mutant and the standard competitor and calculating the concentration or copy number of the rare mutant nucleic acid variant in the original sample base on the known amount of the competitor initially added to the amplification reaction in the step a).

10. The method of claim 9, wherein a mixture of dNTP(s)/ddNTP(s) are used, wherein none of the dNTPs or ddNTPs can also be used for the extension of the wildtype DNA, and the extension product from the rare mutant and the control DNA can be distinguished.

11. The method of claim 9, wherein the consumption of ddNTPs is quantified.

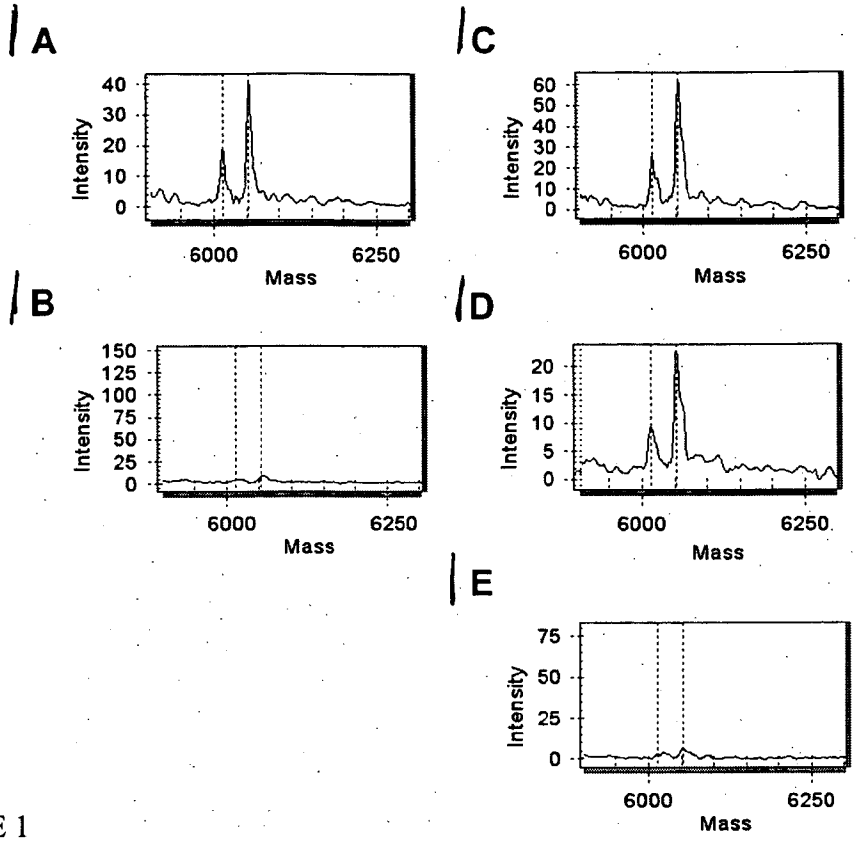
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FIGURE 1

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Charles R. Cantor and Chunming Ding
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For: DETECTION AND QUANTIFICATION OF RARE MUTATIONS

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APPLICATION DATA SHEET
37 C.F.R. § 1.76

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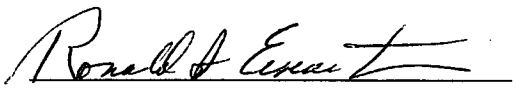
Extent of interest of assignee in application: entire

Date: 2/18/04

Respectfully submitted,



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